[CANCER RESEARCH 56, 1104–1110, March 1, 1996]

Induction of Autologous Tumor Killing by Heat Treatment of Fresh Human Tumor Cells: Involvement of γδ T Cells and Heat Shock Protein 70

Yu-quan Wei, Xia Zhao, Yoshitaka Kariya, Hideki Fukata, Keisuke Teshigawara, and Atsushi Uchida[1]

Department of Late Effect Studies, Radiation Biology Center, Kyoto University, Yoshida-Konoecho, Sakyo-ku, Kyoto 606-01, Japan

ABSTRACT

Autologous tumor killing (ATK) has been implicated as an important prognostic factor in cancer patients since the ability of blood lymphocytes to kill freshly isolated autologous tumor cells was strongly associated with good prognosis of the patients. The present study was designed to induce or enhance ATK sensitivity of fresh human tumor cells by heat stress. Brief exposure of fresh human tumor cells to elevated temperature increased their susceptibility to lysis by autologous blood lymphocytes in a short-term ^{51}Cr release assay. In addition, the heat-elevated ATK sensitivity was confirmed by clonogenic assays. An increase in ATK was observed with unstimulated lymphocytes in 42% of the cases and OK432 (streptococcal preparation)-activated lymphocytes in 80% of the cases. Stimulation of blood lymphocytes with autologous, heat-stressed tumor cells and OK432 resulted in an increase in number of γδ T cells, which was associated with elevated ATK activity against the stressed tumor cells. At the clonal level, three γδ T-cell clones (Vγ9/Vδ2+) proliferated in response to autologous, heat-stressed tumor cells and/or OK432 and exhibited elevated cytotoxicity against the tumor cells. Western blot analysis revealed an increased expression of heat shock protein (HSP) 70 in heat-treated tumor cells. Some of them expressed HSP70 on their surfaces. The elevated cytotoxicity against heat-stressed tumor cells was inhibited by treatment of targets with anti-HSP70 monoclonal antibody (mAb) or of effector cells with anti-Vδ2 mAb. Reactivity of γδ T cells to autologous, heat-stressed tumor cells was also inhibited by anti-HSP70 mAb. These results indicate that exposure to heat of tumor cells induces ATK susceptibility, especially to OK432-activated effector cells, and suggest that γδ T cells may be involved in ATK against stressed tumor cells through recognition of HSP70 on the target cells.

INTRODUCTION

For a better understanding of the antitumor response of lymphocytes in human cancer patients, cytotoxicity assays with an autologous combination of fresh effector and target cells have been performed (1–6). Blood lymphocytes from 5 to 80% of human cancer patients, depending on the histological types of tumors and metastatic status, expressed ATK[2] activity (1–5). ATK activity tested at the time of surgery was correlated strongly with a postoperative clinical course (1–5). More than 80% patients with positive results in ATK tests at the time of surgery remained tumor-free and alive more than 5 years after curative operation, whereas all ATK-negative patients developed local and/or distant metastases by 2 years and died by 5 years (1–3). The strong correlation of ATK activity with disease-free interval and total survival indicate that ATK activity is a meaningful prognostic indicator, suggest that ATK lymphocytes may be the main effector in the immunological defense system against growth and metastasis of tumor, and provide evidence for immunological control of tumor growth and metastasis. (1–3). In clinical trials, the induction of ATK activity by biological response modifiers has improved the clinical outcome in cancer patients who naturally have no such potential (2, 3). Thus, considerable emphasis should be placed on a strategy that induces ATK activity *in vivo*.

HSP is known to be a highly conserved protein from prokaryote to eukaryote and to be induced in cells by various types of stress, such as increased temperature (7–12). Immunodominant antigens from a wide variety of bacteria and parasites were identified as belonging to HSP families (9). HSP expression increased in virus-transformed, chemically induced, and oncogene-transfected tumor cells (8–12). Tumor-rejection antigens expressed on some mouse tumors were identified as HSP (8, 11–14). Members of HSP70 family are suggested to be involved in antigen processing and presentation through binding to short peptides (8, 15–18), and the putative peptide binding site of HSP70 appears to be similar to that already determined for the peptide binding domains of MHC (7, 17, 18). In addition, two genes encoding HSP70 were mapped within MHC (18).

A subset of T cells express γδ TCR and have limited variable (V) region repertoire. The nature of antigens recognized by γδ T cells and precise biological functions of γδ T cells are not fully understood yet (19–21). Recently, considerable attention has been paid to the recognition of HSP by γδ T cells (9, 19–21). Both murine and human γδ T cells responded to mycobacterial extracts, and M_r 65,000 mycobacterial HSP and its peptides stimulated γδ T cells (9). The AGroEL homologue on Daudi Burkitt's lymphoma was recognized by γδ T cells (9).

The present study was designed to investigate whether heat stress induces ATK sensitivity and whether γδ T cells are involved in the response through interaction with HSP expressed on tumor cells.

MATERIALS AND METHODS

Patients and Treatment with OK432. This study included 36 patients with previously untreated, localized neoplasms: 12 mucous or serous adenocarcinomas of ovary; 3 dysgerminomas of ovary; 3 cases of adenocarcinomas of lung; 5 squamous cell carcinomas of lung; 5 adenocarcinomas of stomach; 3 squamous cell carcinomas of esophagus; and 5 hepatocellular carcinomas. Five of them, including 3 gastric adenocarcinomas of stomach, 1 hepatocellular carcinoma, and 1 squamous cell carcinoma of lung, were treated with i.v. injections of 500 μg streptococcal preparation OK432 (Chugai Pharmaceutical, Tokyo, Japan; Ref. 4) 7 and 3 days prior to surgery. The 26 female and 10 male patients ranged in age from 32 to 75.

Blood Lymphocytes and Treatment with OK432. Effector cells were prepared from peripheral blood as described (4, 6). Lymphocyte-rich mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients, washed, and suspended in RPMI 1640 supplemented with 25 μM HEPES, 2 mM L-glutamine, 100 units penicillin/ml, 100 μg streptomycin/ml, and 10% heat-inactivated human AB serum, and further purified by adherence to serum-coated plastic dishes. In some experiments, lymphocytes (2×10^6/ml) were treated *in vitro* alone or with 10 μg OK432/ml for 24 h at 37°C.

Tumor Cells. Specimens of tumor tissues were obtained from cancer patients at the time of surgery. Tumor cells were isolated from the specimens by enzymatic treatment with collagenase (1 μg/ml) and DNase (0.2 μg/ml), followed by centrifugation on discontinuous three-step (10, 15, and 25%) Percoll gradients and two-step (75 and 100%) Ficoll-Hypaque gradients and by adherence to plastic surfaces (1, 4). The tumor-enriched fraction contained more than 90% tumor cells as judged by morphological examination of Wright-Giemsa-stained smears. More than 90% of the tumor cells was viable, according to the trypan blue dye exclusion test.

Induction of HSP. ^{51}Cr-labeled or unlabeled tumor cells at a concentration of 2×10^5/ml in complete medium were incubated in a water bath set at 42°C

Received 3/20/95; accepted 1/4/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: ATK, autologous tumor killing; HSP, heat shock protein; mAb, monoclonal antibody; TCR, T-cell receptor; IL, interleukin; E:T, effector:target ratio.

for 30–60 min, as described (20). Control samples were incubated in parallel in a bath at 37°C. After heat treatment, the cells were incubated at 37°C for 2 h in a humidified 5% CO_2 atmosphere.

mAb. Leu4 (ant-CD3), Leu2a (anti-CD8), Leu3a (anti-CD4), Leu19 (anti-CD56), Leu11c (anti-CD16), and anti-TCR-γ/δ-1 were obtained from Becton Dickinson (Mountain View, CA). Diversi-T Vδ2 (recognizing Vδ2) was obtained from T Cell Diagnostics (Cambridge, MA), and anti-Vγ9 was from Immunotech SA (Marseilles, France). Anti-HLA-ABC mAb (W6/32) was from Dako (Copenhagen, Denmark). Anti-HSP25 (clone no. IAP-9) and anti-HSP70 (clone no. BRM-22) mAb were obtained from Sigma Chemical Co., and anti-HSP90 mAb was from Funakoshi (Tokyo, Japan). Purified HSP70 was from Sigma. Optimal concentrations of mAb were used in the present study: anti-Vδ2, 3 μg/ml; anti-HSP25, 5 μg/ml; anti-HSP70, 5 μg/ml; anti-HSP90, 5 μg/ml; and anti-HLA-ABC, 5 μg/ml.

Expansion and Cloning of γδ T Cells. Autologous mixed lymphocytes-tumor cultures were performed using 24-well, flat-bottomed tissue culture plates with 1×10^6 blood lymphocytes and 1×10^5 irradiated heat-treated autologous tumor cells in a total volume of 1 ml complete medium, as described previously (1, 6). After 3 days of culture, OK432 was added at a concentration of 10 μg/ml and was cultured for an additional 4 days. These lymphocytes cultured with heat-treated tumor cells and OK432 were seeded at 1 cell/well in a round-bottomed well of microtiter plates preseeded with 3×10^3 irradiated, autologous heat-treated tumor cells, 1×10^4 blood mononuclear cells, and 20 units IL-2/ml. One week later, 100 μl supernatant were removed and replaced by 100 μl fresh medium with stimulator and feeder cells. Proliferating microcultures were then transferred into a flat-bottomed well of microtiter plates preseeded with stimulators and IL-2 for another week. The proliferating microcultures were transferred into 24-well culture plates, and proliferating lymphocytes were expanded by the addition of stimulator cells and IL-2.

Proliferation Assay. Proliferation of cells was determined in a [^{3}H]thymidine incorporation assay, as described (1, 4). Briefly, 2.5×10^4 cells were cultured alone or with heat-treated or untreated autologous tumor cells in the presence or absence OK432 in a total volume of 0.2 ml for 72 h. One-half μCi [^{3}H]thymidine was added for the last 8 h of the culture. Cells were then harvested, and incorporation of [^{3}H]thymidine was counted with a liquid scintillation counter. Results were expressed as mean cpm. In proliferation inhibition experiments, different concentrations of anti-HSP25, anti-HSP70, and anti-HSP90 mAb were added to the culture.

Cytotoxicity Assay. A 6-h ^{51}Cr release assay was performed as described (1, 6). Briefly, 100 μl of ^{51}Cr-labeled target cells and effector cells were assigned at different E:T ratios to each well of microtiter plates and incubated for 6 h at 37°C. Samples were then harvested, and the activity was counted in an autogamma scintillation counter. The percentage cytotoxicity was calculated by:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

In cytotoxicity inhibition assays, effector cells or ^{51}Cr-labeled tumor cells were pretreated with mAb for 30 min at room temperature, washed, and tested (12). Effector cells were treated with anti-Vδ2 mAb, and tumor cells were with anti-HSP mAb. Control assays were performed in the presence of irrelevant mouse IgG mAb.

Clonogenic Assay. Tumor clonogenic assays were performed as described (22). Briefly, a bottom layer of 0.5% agarose was prepared in 35-mm plastic dishes. After solidification, 1 ml cell suspensions containing tumor cells alone or with lymphocytes at an E:T of 20:1 were layered on the top. The dishes were then incubated for 12 days at 37°C in a humidified 5% CO_2 atmosphere. After culture, colonies were counted with an inverted microscope, and growth inhibition of clonogenic tumor cells was calculated by:

$$\% \text{ growth inhibition} = 1 - \frac{\text{Experimental tumor colonies}}{\text{Control tumor colonies}} \times 100$$

where control tumor colonies were obtained by counting the number of tumor cell colonies derived from corresponding unstressed tumor cells in the absence of lymphocytes. All experiments were done in duplicate.

Western Blot Analysis. Western blot analysis was performed as described (10). Briefly, 1×10^6 tumor cells were lysed in 200 μl lysis buffer containing 50 mM Tris-buffered HCL, 1% NP40, 1% SDS, 1 μM leupeptin, 1 μM pepstatin, 100 μM EDTA, 100 μM phenylmethysulfony fluoride, and 0.5% Triton X-100, and the protein concentration was determined by a bicinchoninic acid protein assay reagent. The samples were denatured in sample buffer containing 0.025 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue and boiled at 100°C for 5 min, and proteins were separated by SDS-PAGE (12.5% polyacrylamide) at 50 mA for 1 h. Gels were electroblotted for 1 h at 2.5 mA/cm^2 with Sartoblot onto a polyvinylidene difluoride membrane. The membrane blots were rinsed with TTBS and blocked by 3% gelatin. The blots were incubated first with anti-HSP mAb and then with a biotinylated second antibody, followed by transfer to Vectastain ABC. 3,3′-Dimethylaminoazobenzene substrate kits for horseradish peroxidase (Vector Laboratories) was used for the development of color. The prestained standard of Bio-Rad was used as a reference molecular size standard. In some experiments, plasma membranes isolated from tumor cells (23) were subjected to Western blot analysis.

Flow Cytometry. Flow cytometric analysis was performed using a FACScan (Becton Dickinson), as described (24). Tumor cells were stained with anti-HSP25, anti-HSP70, and anti-HSP90 mAb, and then were labeled with FITC-conjugated F(ab′)$_2$ fragment of goat antimouse IgG. Dead cells were excluded on the basis of forward and side light scatter and by propidium iodide staining. The surface phenotype of lymphocytes was determined in one- or two-color flow cytometry after staining with fluorescein- or phycoerythrin-labeled mAb or by indirect immunofluorescence staining.

RESULTS

Induction of ATK Sensitivity by Heat Treatment of Tumor Cells. Blood lymphocytes exhibited various levels of cytotoxicity against freshly isolated, autologous tumor cells in ^{51}Cr release assays. In an attempt to evaluate the effect of heat stress on tumor susceptibility to ATK, tumor cells were exposed to an elevated temperature of 42°C for 30 min prior to cytotoxicity assays. ATK was higher against heat-stressed tumor cells than against unstressed ones (Table 1). ATK sensitivity was induced and augmented in 11 of 26 (42%) samples. *In vitro* treatment of lymphocytes with OK432 resulted in a further increase in cytotoxicity against autologous heat-treated tumor cell as well as untreated cells. When these activated lymphocytes were used as effectors, an induction or elevation of ATK sensitivity was observed with 20 of 25 (80%) heat-stressed tumor samples. No increase in spontaneous ^{51}Cr release was seen with heat-stressed tumor cells. By contrast, heat stress of the K562 erythroleukemia cell line or normal fibroblasts did not affect their sensitivity to lysis by natural killer and activated killer cells (data not shown). The increased ATK sensitivity detected in ^{51}Cr release assays was strongly associated with an increased number of dead cells determined by trypan blue dye exclusion tests ($\gamma = 0.95$; data not shown).

Next, the effect of heat stress on tumor clonogenicity was determined in clonogenic assays. Tumor cells treated by heat alone maintained the potential to form colonies (Fig. 1). However, when the stressed tumor cells were cocultured with untreated or OK432-treated autologous lymphocytes, their potential to form colonies was inhibited ($P < 0.01$).

A possible involvement of HSP in the heat-induced ATK was then considered. Results of a representative experiment are shown in Fig. 2. Treatment of heat-stressed tumor cells with mAb against HSP70 abrogated heat-induced cytotoxicity, while anti-HSP25 or anti-HSP90 mAb had no effect. These results indicate that heat stress induces HSP70 in tumor cells, which may be involved in the enhanced ATK sensitivity.

Augmentation of ATK Activity against Stressed Tumor Cells by Administration of OK432. Since *in vitro* treatment of lymphocytes with OK432 enhanced cytotoxicity against autologous, heat-

Table 1 *Induction of ATK sensitivity by heat treatment of tumor cells*

Tumor cells were incubated at 37°C or 42°C for 30 min and tested for % cytotoxicity by autologous, OK432-treated and untreated blood lymphocytes at E:T of 40:1 and 20:1 in 6-h ^{51}Cr release assays. Results are expressed as mean % cytotoxicity ± SD. Mean spontaneous ^{51}Cr release of unstressed and stressed 26 tumor samples was 19.6 ± 4.5% and 20.1 ± 4.1% of the total isotope count, respectively.

Case no.	E/T	% Cytotoxicity of lymphocytes			
		PBL[a]		OK432-activated PBL	
		Unstressed	Stressec	Unstressed	Stressed
1	40:1	−3.7 ± 0.3	17.9 ± 0.9[b]	18.4 ± 3.8	29.3 ± 2.0[b]
	20:1	−2.5 ± 0.9	11.8 ± 1.0[b]	11.3 ± 1.2	20.2 ± 2.2[b]
2	40:1	1.7 ± 1.9	1.7 ± 2.0	13.3 ± 2.7	26.1 ± 2.6[b]
	20:1	1.6 ± 2.4	1.8 ± 1.2	10.5 ± 3.2	20.1 ± 1.5[b]
3	40:1	1.1 ± 1.9	2.4 ± 0.8	4.1 ± 1.5	14.5 ± 0.9[b]
	20:1	1.2 ± 1.6	2.1 ± 1.3	3.4 ± 2.3	10.2 ± 1.8[b]
4	40:1	1.3 ± 1.1	0.2 ± 0.4	1.2 ± 0.2	0.7 ± 1.1
5	40:1	17.4 ± 3.3	17.2 ± 4.6	33.3 ± 6.8	30.0 ± 5.8
	20:1	10.4 ± 2.6	12.3 ± 4.5	24.7 ± 5.6	22.5 ± 4.3
6	40:1	3.4 ± 2.7	1.2 ± 1.5	10.7 ± 3.0	22.5 ± 0.2[b]
	20:1	2.1 ± 1.9	3.2 ± 1.3	5.6 ± 1.5	15.3 ± 1.4[b]
7	40:1	3.3 ± 2.5	7.2 ± 3.5	3.1 ± 0.9	15.4 ± 2.5[b]
	20:1	−1.1 ± 1.5	2.3 ± 1.4	1.2 ± 2.2	10.1 ± 1.1[b]
8	40:1	5.0 ± 5.2	3.4 ± 4.9	12.7 ± 2.9	20.3 ± 0.8[b]
	20:1	1.2 ± 2.5	2.3 ± 3.3	5.4 ± 2.7	14.6 ± 1.7[b]
10	40:1	2.7 ± 0.2	15.1 ± 0.6[b]	13.6 ± 1.8	29.6 ± 3.5[b]
	20:1	1.2 ± 0.7	10.2 ± 2.3[b]	16.4 ± 0.6	25.1 ± 2.1[b]
11	40:1	1.1 ± 1.2	1.4 ± 0.9	1.1 ± 0.9	1.1 ± 1.2
	20:1	−1.2 ± 1.4	1.8 ± 2.1	1.5 ± 1.7	1.6 ± 1.9
12	40:1	2.8 ± 2.9	22.9 ± 3.6[b]	6.5 ± 1.1	26.2 ± 3.4[b]
	20:1	1.2 ± 2.7	14.6 ± 2.3[b]	3.2 ± 1.2	16.3 ± 2.6[b]
13	40:1	3.8 ± 0.7	3.6 ± 1.0	6.8 ± 1.6	14.9 ± 1.1[b]
	20:1	0.8 ± 1.0	0.7 ± 1.6	1.2 ± 1.1	10.2 ± 0.8[b]
14	40:1	3.9 ± 3.2	23.1 ± 2.8[b]	22.0 ± 4.7	33.1 ± 1.1[b]
	20:1	2.4 ± 1.9	16.2 ± 1.7[b]	13.2 ± 3.8	25.4 ± 1.3[b]
15	40:1	12.7 ± 1.3	20.0 ± 4.5[b]	17.6 ± 1.1	26.6 ± 1.9[b]
	20:1	6.4 ± 2.3	12.4 ± 2.1[b]	11.2 ± 2.6	19.3 ± 1.4[b]
16	40:1	0.8 ± 0.6	10.2 ± 2.3[b]	1.2 ± 1.4	15.0 ± 4.2[b]
17	40:1	1.9 ± 2.3	11.2 ± 0.7[b]		
20	40:1	5.7 ± 0.5	4.1 ± 1.1	10.1 ± 1.3	17.1 ± 1.7[b]
	20:1	1.3 ± 1.5	2.7 ± 2.6	4.6 ± 1.1	10.2 ± 1.4[b]
22	40:1	5.0 ± 0.7	3.3 ± 2.3	7.1 ± 0.5	19.8 ± 1.4[b]
	20:1	2.3 ± 1.2	2.5 ± 1.7	2.3 ± 1.8	12.1 ± 0.9[b]
25	40:1	5.1 ± 2.7	13.2 ± 3.3[b]	25.7 ± 1.4	42.6 ± 3.3[b]
26	40:1	−0.8 ± 0.9	0.1 ± 0.6	−0.4 ± 0.4	1.9 ± 1.1
	20:1	0.1 ± 1.2	2.1 ± 1.4	1.1 ± 0.9	0.9 ± 1.9
27	40:1	−1.8 ± 2.6	6.3 ± 2.3	0.4 ± 0.7	17.8 ± 1.8[b]
	20:1	1.2 ± 2.4	4.6 ± 2.3	2.3 ± 2.1	12.2 ± 1.3[b]
29	40:1	−1.7 ± 0.5	3.5 ± 0.7	0.1 ± 0.2	13.3 ± 0.6[b]
30	40:1	14.1 ± 1.9	26.3 ± 4.0[b]	21.4 ± 4.3	34.8 ± 3.1[b]
	20:1	7.2 ± 2.3	16.3 ± 2.0[b]	13.2 ± 2.1	25.2 ± 2.1[b]
33	40:1	12.3 ± 1.1	20.6 ± 1.6[b]	17.5 ± 3.2	26.4 ± 1.4[b]
	20:1	5.6 ± 2.1	14.2 ± 1.2[b]	10.3 ± 3.1	21.2 ± 1.5[b]
34	40:1	12.2 ± 2.0	19.2 ± 2.2[b]	26.9 ± 4.0	50.6 ± 3.2[b]
	20:1	7.3 ± 1.5	15.3 ± 1.3[b]	17.2 ± 2.7	39.4 ± 4.8[b]
36	40:1	1.0 ± 1.2	1.0 ± 1.5	12.3 ± 2.3	14.3 ± 3.4
	20:1	1.1 ± 1.4	1.7 ± 1.6	8.7 ± 2.4	9.3 ± 1.3

[a] PBL, peripheral blood lymphocytes.
[b] Values are significantly different from those of unstressed tumor cells according to Student's t test at $P < 0.05$.

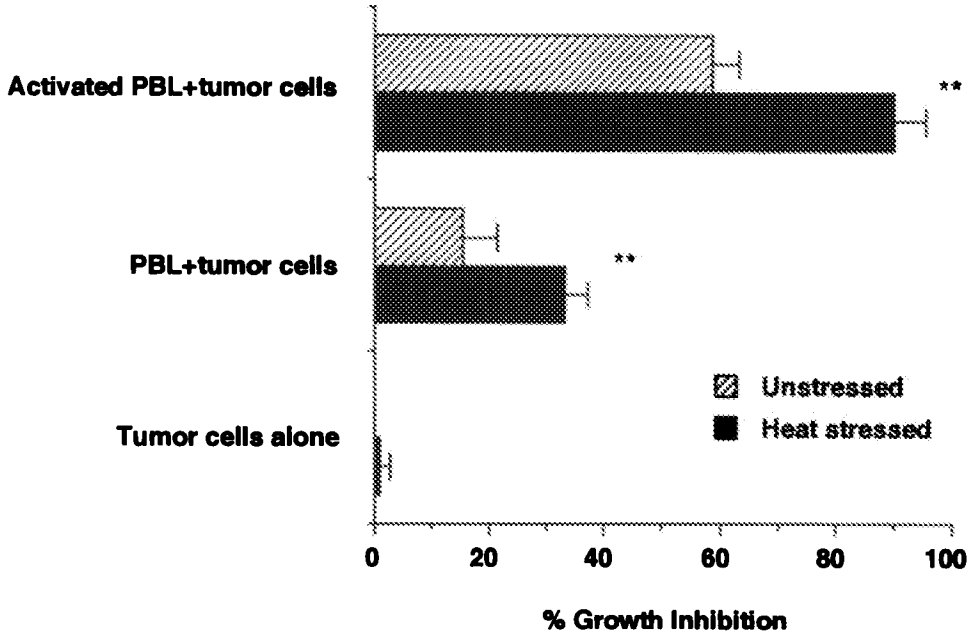

Fig. 1. Augmentation by heat stress of tumor cells of sensitivity to growth-inhibitory activity of autologous lymphocytes. Fresh tumor cells of a patient with serous adenocarcinoma of the ovary were treated with heat and tested for clonogenic potential in the presence or absence of autologous OK432-treated and untreated blood lymphocytes at an E:T of 20:1. Results are expressed as % growth inhibition. **, values are significantly different from those of unstressed cells at $P < 0.01$. Similar results were obtained in four different cases; *bars*, SD.

stressed tumor cells, we explored the possibility that *in vivo* administration of OK432 activates blood lymphocytes in human cancer patients. To this end, cancer patients were treated with i.v. injections of OK432. Blood lymphocytes from OK432-treated patients exhibited higher cytotoxicity against heat-treated autologous tumor cells than those from corresponding patients before therapy (Table 2).

Expansion of γδ T Cells by Stimulation with Autologous Heat-stressed Tumor Cells and OK432. Stimulation of blood lymphocytes with heat-stressed autologous tumor cells increased γδ T cells in 7-day mixed cultures (Fig. 3*a*). A similar increase in γδ T cells was obtained by stimulation with OK432 alone. Simultaneous stimulation with autologous heat-stressed tumor cells and OK432 produced the highest numbers of γδ T cells ($P < 0.05$). Along with an increase in γδ T cells, ATK activity against heat-treated tumor cells increased in mixed cultures (Fig. 3*b*). The highest killing of autologous stressed tumor cells was seen with lymphocytes that were activated by a combination of autologous heat-stressed tumor cells and OK432 ($P < 0.05$). By contrast, these activated lymphocytes showed no increased ATK against untreated tumor cells. These results suggest that γδ T cells may be involved in the killing of autologous heat-treated tumor cells.

Table 2 *Induction of ATK activity by in vivo* administration of OK432

Cancer patients were treated with OK432, and blood lymphocytes were obtained before and after OK432 therapy and were tested for cytotoxicity against autologous heat-stressed and unstressed tumor cells. Results are expressed as mean % cytotoxicity ± SD at an E:T of 40:1.

Case no.	Targets	% Cytotoxicity of lymphocytes	
		Before therapy	After OK432 therapy
9	Unstressed	3.4 ± 2.2	13.8 ± 1.6
	Stressed	7.0 ± 2.0	26.7 ± 4.7[a,b]
18	Unstressed	1.5 ± 2.1	4.6 ± 1.2
	Stressed	5.0 ± 4.5	3.8 ± 2.8
19	Unstressed	10.2 ± 1.7	21.8 ± 0.8
	Stressed	20.2 ± 0.3[a]	33.5 ± 2.0[a,b]
23	Unstressed	15.2 ± 1.3	23.1 ± 1.8
	Stressed	24.5 ± 4.8[a]	40.9 ± 3.9[a,b]
24	Unstressed	3.8 ± 0.6	12.8 ± 0.9
	Stressed	13.8 ± 1.8[a]	22.3 ± 1.2[a,b]

[a] Values are significantly different from those of unstressed tumor cells according to student's *t* test at $P < 0.05$.

[b] Values are significantly different from those of lymphocytes before therapy according to student's *t* test at $P < 0.05$.

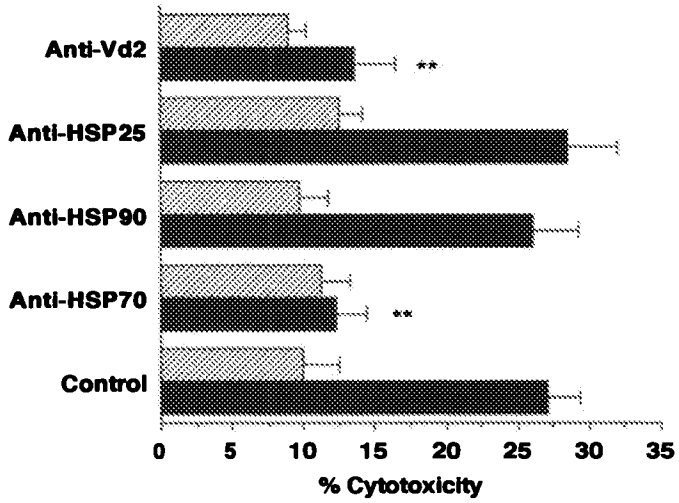

Fig. 2. Inhibition of heat-induced ATK by anti-HSP70 mAb and anti-Vδ2 mAb. ^{51}Cr-labeled stressed (■) and unstressed tumor cells (▨) were treated with anti-HSP25, anti-HSP70, and anti-HSP90 mAb prior to a cytotoxicity assay. OK432-activated lymphocytes were pretreated with anti-Vδ2 mAb. Results are expressed as % cytotoxicity at an E:T of 40:1. **, values are significantly different from those of control at $P < 0.05$. Similar results were obtained in three different cases; *bars*, SD.

ATK against Heat-treated Tumor Cells by γδ T-Cell Clones. T-cell clones were established from mixed cultures of blood lymphocytes, autologous heat-treated tumor cells, and OK432 by limiting dilution techniques. Three of them, termed 1F3, 1G2, and 2B5, were CD3+TCRγδ+Vγ9+Vδ2+CD8−CD4−CD16−, whereas the other six clones were CD3+CD8+CD4−TCRγδ−. γδ T-cell clones proliferated in response to autologous heat-treated tumor cells and OK432 (Fig. 4*a*). This proliferation was abrogated by anti-HSP70 mAb but not by anti-HSP25 or anti-HSP90 mAb. Anti-TCR-γ/δ-1 mAb also stimulated the proliferation of these clones.

Three γδ T-cell clones exhibited cytotoxicity against autologous, heat-treated tumor cells (Fig. 4*b*). Treatment with anti-HSP70 mAb of stressed tumor cells reduced their sensitivity to autologous γδ T-cell clones.

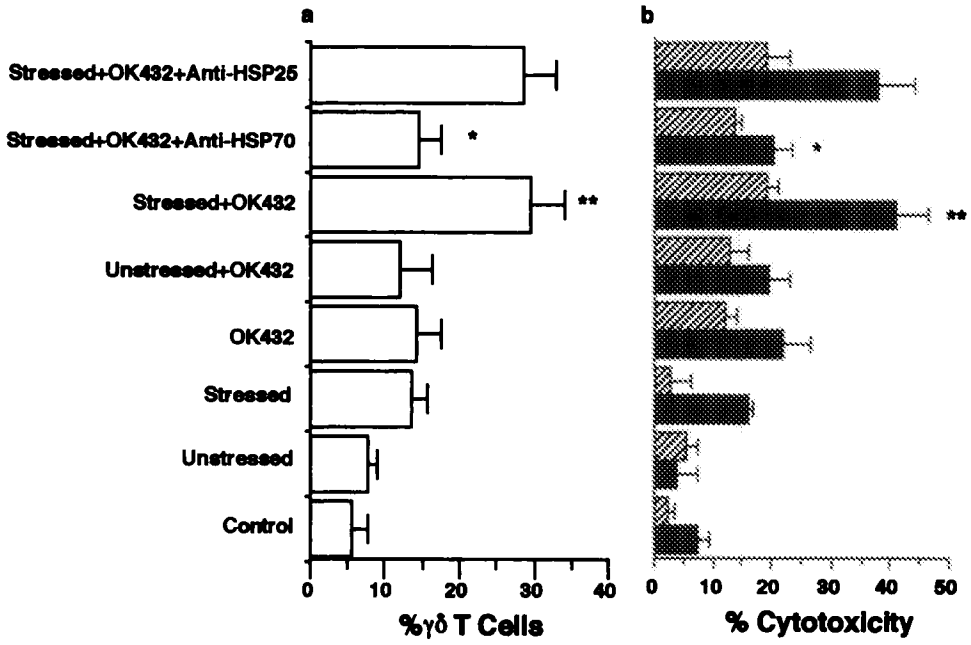

Fig. 3. Expansion of γδ T cells and induction of ATK activity by stimulation with autologous heat-treated tumor cells and OK432. Lymphocytes were cultured with autologous heat-treated tumor cells and/or OK432 for 7 days in the presence or absence of anti-HSP25 and anti-HSP70 mAb. Then cultured cells were measured for the number of γδ T cells (*a*) and cytotoxicity against autologous tumor cells (*b*). Results are expressed as the means of triplicate tests; *bars*, SD. **, values are significantly different from those of other stimulation at $P < 0.01$. *, values are significantly different from those of stressed plus OK432 at $P < 0.01$. ■, stressed tumor cells; ▨, unstressed tumor cells. Similar results were obtained in three different experiments.

Similarly, the activity of γδ T-cell clones was abrogated by treatment with anti-Vδ2 mAb prior to a cytotoxicity assay. By contrast, anti-HLA-class I mAb had no effect on ATK. These γδ T-cell clones failed to kill untreated and heat-treated K562. On the other hand, TCRγδ-negative T-cell clones showed no elevated cytotoxicity against stressed tumor cells (data not shown). These results indicate at the clone level that γδ T cells may recognize and kill autologous, heat-stressed tumor cells through interaction of HSP70 induced in the targets by heat.

Induction of HSP70 by Heat Treatment. To confirm the HSP70 induction by heat stress of tumor cells, we used Western blot and flow cytometry techniques. As shown in Fig. 5, positive staining was seen in *Lane A* containing 5×10^4 stressed tumor cells but not in *Lane B* containing the same number of unstressed tumor cells. When 1×10^5 tumor cells were applied, positive bands were also observed with unstressed tumor cells, the intensity of which was, however, lower than the stressed tumor cells. Western blot analysis of separated plasma membrane fractions suggested an expression of HSP70 on heat-stressed tumor cells but not on unstressed tumor cells (Fig. 6). In flow cytometry analysis, a proportion (3–35%) of heat-treated tumor cells expressed HSP70 on their surface, whereas untreated cells did not (Fig. 7).

It might be possible that a small fraction of tumor cells were lysed and intracellular HSP70 had, in turn, become associated with the surface of intact cells, resulting in positive staining for HSP70. To exclude the possibility, we incubated unstressed tumor cells with supernatants of heat-stressed tumor cells or suspensions containing exogenous purified HSP70 (1–100 μg/ml) for 1–5 h. No unstressed cells were stained for HSP70, indicating that positive staining is not a result of nonspecific binding of HSP70 from extracellular sources. Furthermore, heat-induced ATK sensitivity was closely associated with an increased number of HSP70-positive cells ($\gamma = 0.74$ for untreated effector cells, and $\gamma = 0.94$ for OK432-treated effectors; Fig. 8). In addition, other stress proteins, including HSP25 and HSP90, were not modified by the heat of tumor cells. Also, the expression of HLA class I and intercellular adhesion molecule-1 molecules was not altered by exposure to heat (data not shown). These results indicate that exposure to heat of fresh human tumor cells

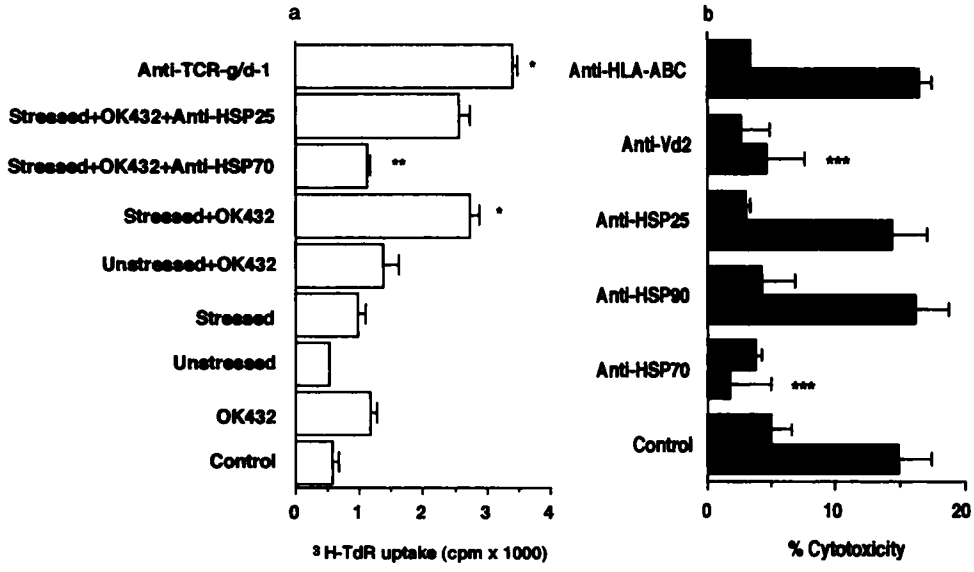

Fig. 4. Proliferation and cytotoxicity of γδ T-cell clone to autologous stressed tumor cells and their blocking by anti-HSP70 mAb. IG2 γδ T-cell clone of a patient with dysgerminoma of the ovary was tested for proliferative capacity to autologous tumor cells and OK432 (*a*) and cytotoxicity against autologous untreated (■) and stressed tumor cells (■) at an E:T of 20:1 (*b*). In inhibition experiments, tumor cells were treated with mAb against HSP25, HSP70, HSP90, and HLA-ABC, and effector cells were treated anti-Vδ2 mAb. *, values are significantly different from those of controls at $P < 0.01$. **, values are significantly different from those of stressed plus OK432 at $P < 0.01$. ***, values are significantly different from those of control at $P < 0.01$. Similar results are obtained in 2 γδ T-cell clones.

induces HSP70, which may be involved in the elevated sensitivity to lysis by autologous lymphocytes.

DISCUSSION

Several observations have been made in the present study concerning heat stress, HSP, and ATK. The present study has first demonstrated that a brief exposure to heat of freshly isolated human tumor cells induces or enhances their susceptibility to lysis by autologous lymphocytes. This is further confirmed by tumor clonogenic assays in

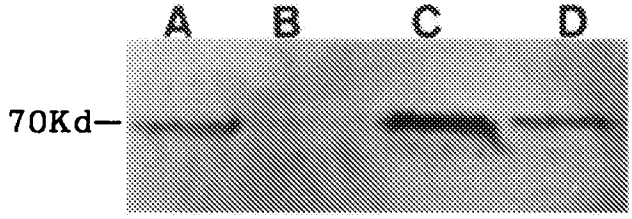

Fig. 5. Expression of HSP70 in heat-treated tumor cells (extracts from total tumor cells) from adenocarcinoma of lung determined by Western blot analysis. *Lane A*, 2×10^4 heat-stressed tumor cells. *Lane B*, 2×10^4 unstressed tumor cells. *Lane C*, 1×10^5 stressed tumor cells. *Lane D*, 1×10^5 unstressed tumor cells. *70Kd*, M_r 70,000.

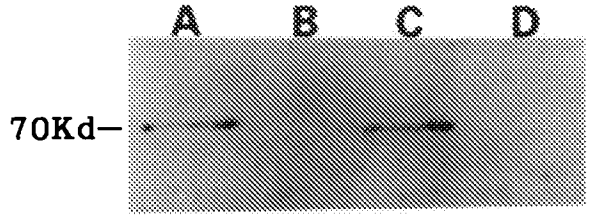

Fig. 6. Expression of HSP70 on cell membrane of heat-stressed tumor cells determined by Western blot analysis. Tumor cells were obtained from a patient with dysgerminoma of the ovary (*Lanes A* and *B*) and adenocarcinoma of the lung (*Lanes C* and *D*), and cell membrane extracts from 1×10^6 tumor cells were loaded to each lane. *Lane A*, heat-stressed; *Lane B*, unstressed; *Lane C*, heat-stressed; *Lane D*, unstressed. *70Kd*, M_r 70,000.

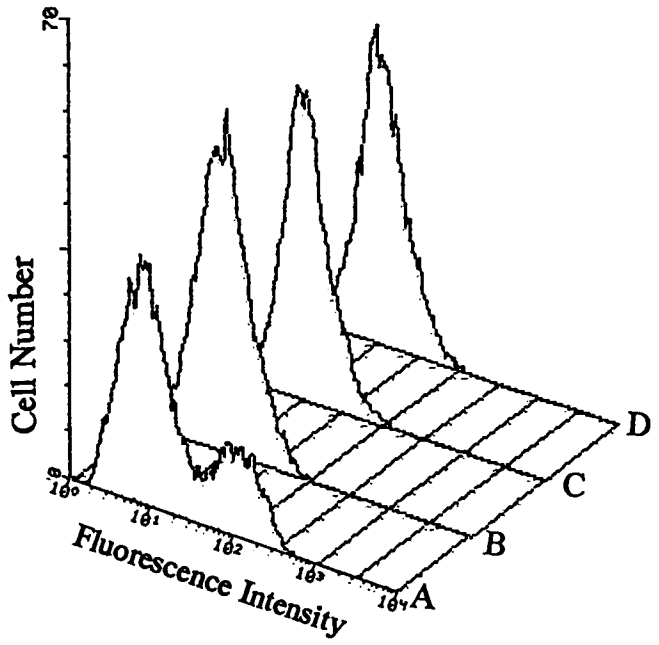

Fig. 7. Expression of HSP 70 on the surface of heat-stressed tumor cells analyzed by flow cytometry. Heat-stressed (*A*) and unstressed (*C*) tumor cells from a patient with mucous adenocarcinoma of the ovary were stained with anti-HSP70 mAb, followed by FITC-conjugated $F(ab')_2$ fragments of goat antimouse IgG. Stressed (*B*) and unstressed (*D*) tumor cells were stained with secondary mAb alone as control.

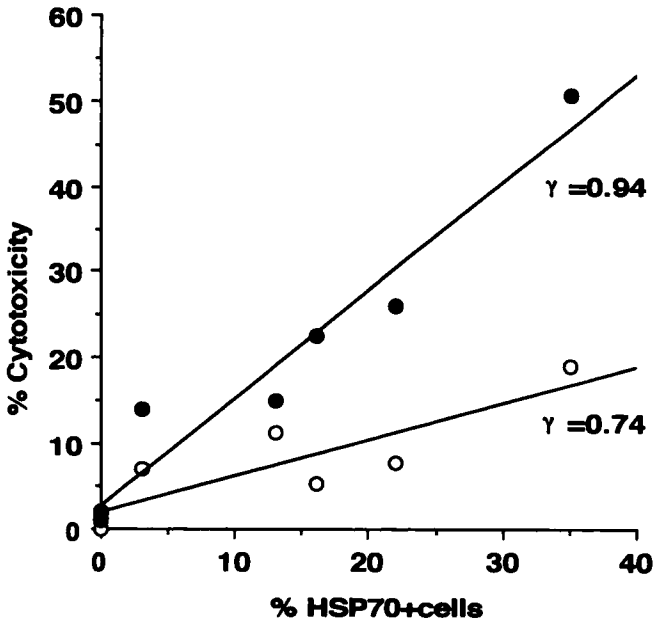

Fig. 8. Relationship between frequency of HSP70-positive cells and ATK sensitivity of heat-treated tumor cells. Untreated (○) or *in vitro* OK432-activated lymphocytes (●) were tested against heat-stressed and unstressed tumor cells in 6-h ^{51}Cr release assays at an E:T of 40:1. Results are expressed as means ± SD. Numbers of HSP70-positive cells were determined by flow cytometry. No positive cells were detected among unstressed tumor cells. The frequency of HSP70-positive cells related to augmented ATK by untreated (γ = 0.74) and OK432-activated (γ = 0.94) lymphocytes.

which growth inhibition was observed with heat-treated tumor cells following exposure to autologous lymphocytes. The heat-induced ATK sensitivity is not a result of direct killing of tumor cells by heat since heat-stressed tumor cells showed no increase in spontaneous chromium release or loss of tumor clonogenicity. We have reported previously that the streptococcal protein OK432 induces or augments ATK activity (2, 4). The induction of ATK sensitivity was more frequently observed when lymphocytes were activated *in vitro* by OK432. Blood lymphocytes from cancer patients who received OK432 administration exhibited further elevated ATK activity against heat-stressed tumor cells. Hyperthermia therapy alone has produced only marginally useful results in cancer patients (7). On the other hand, we have reported that ATK activity is closely associated with prognosis of patients with various tumor types, and ATK induction therapy may result in a prolongation of survival (1–3). Taken together, biological therapy with OK432 in combination with hyperthermia might be a new approach for cancer treatment modality.

HSP is induced in cells by various types of stress, including increased temperature, lymphokine activation, virus infection, and attack by reactive oxygen metabolites (7–10). Data presented in this communication have extended these findings to show that a brief exposure to heat induces HSP70 expression in the cytoplasm and surface of fresh human tumor cells. The induction of HSP70 was associated with heat-induced ATK sensitivity, both of which were abrogated by anti-HSP70 mAb. HSP70 has been shown to be recognized by γδ T cells (12, 14). In this respect, γδ T cells are found to proliferate and acquire ATK activity in response to autologous, stressed tumor cells and OK432. This is further confirmed at clonal levels by data indicating that γδ T-cell clones established from the culture showed autologous heat-treated, tumor-restricted proliferation and cytotoxicity. Thus, it seems likely that a brief exposure to heat induces HSP70 in tumor cells, which in turn may cause them to become susceptible to lysis by autologous γδ T cells. In this regard, HSP70 is reported to function as tumor rejection antigens (12, 14) and antigen presentation molecules (15–18).

Two processes may be going on in heat-treated tumor cells: relocalization of HSP70 to the cell surface; and *de novo* synthesis of elevated levels of HSP70, which in turn may migrate to the cell surface. An increase in *de novo* synthesis of HSP70 may not be necessary for an induction of ATK sensitivity by heat if no relocation of HSP70 on the cell surface occurred, since no sensitization to ATK was seen in case nos. 11 and 26 in which HSP70 was not expressed on cell surfaces by heat shock treatment of tumor cells, although they had elevated synthesis of HSP70. In addition, preliminary experiments by the use of cycloheximide and actinomycin D revealed that ATK sensitivity was induced in tumor samples that were treated with these RNA and protein synthesis inhibitors. By contrast, blocking of HSP70 expression by anti-HSP70 mAb abolished the induction of ATK sensitivity by heat. Furthermore, the number of tumor cells expressing HSP70 on their surface was closely associated with the degree of ATK sensitization. It seems thus evident that relocation of HSP70 on the cell surface is prerequisite for the induction of ATK sensitivity.

It is known that heat treatment leads to a redistribution of HSP70 within the cell. In the present study, HSP70 was expressed on the surface of heat-stressed tumor cells when determined by flow cytometry. It seems possible that a small fraction of tumor cells was lysed, and the intracellular HSP70 has now become associated with the surface of intact cells. The possibility, however, could be ruled out since no HSP70 was detected on unstressed tumor cells, even when they were cocultured with supernatants of heat-stressed tumor cells or exogenous purified HSP70. In addition, Western blot analysis of isolated cell membrane revealed that a HSP70-positive band was seen in lanes containing isolated cell membrane from heat-stressed tumor cells but not from unstressed cells.

The recognition and lysis of heat-treated tumor cells by autologous lymphocytes may be independent on the MHC system for the following reasons: (*a*) MHC class I molecules were not detected in tumor cells from dysgerminoma, even after exposure to heat, while they were sensitive to ATK; and (*b*) lysis of stressed tumor cells by autologous γδ T-cell clones was not inhibited by anti-MHC class I mAb. This is further supported by the finding that stimulation of Vγ9/Vδ 2 T cells by antigens is not restricted by MHC molecules (9).

In conclusion, the data presented in this communication strongly indicate that heat treatment of freshly isolated human tumor cells induces the susceptibility to lysis by autologous lymphocytes, especially by activated γδ T cells. These findings may be of importance to explore further the role of HSP in the control of tumor cells and to enhance the efficacy of hyperthermia by biological therapy.

REFERENCES

1. Uchida, A., Kariya, Y., Okamoto, N., Sugie, S., Fujimoto, T., and Yagita, M. Prediction of postoperative clinical course by autologous tumor-killing activity in lung cancer patients. J. Natl. Cancer Inst., *82:* 1697–1701, 1990.
2. Uchida, A. Review. Biological significance of autologous tumor-killing activity and its induction therapy. Cancer Immunol. Immunother., *37:* 75–83, 1993.
3. Uchida, A. Clinical significance of autologous tumor-killing activity and its induction therapy. Biotherapy, in press, 1995.
4. Uchida, A., and Micksche, M. Lysis of fresh human tumor cells by autologous peripheral blood and pleural effusion lymphocytes activated by OK-432. J. Natl. Cancer Inst., *71:* 672–677, 1983.
5. Vanky, F., Peterffy, A., Book, A., Willems, T., Klein, E., and Klein, G. Correlation between lymphocyte-mediated autotumor reactivities and clinical course. II. Evaluation of 69 patients with lung carcinoma. Cancer Immunol. Immunother., *16:* 17–23, 1983.
6. Slovin, S. F., Lackman, R. D., Ferrone, S., Kiely, P. E., and Mastrangelo, M. J. Cellular immune response to human sarcomas: cytotoxic T cell clones reactive with autologous sarcomas. I. Development, phenotype, and specificity. J. Immunol., *137:* 3042–3049, 1986.
7. Carper, S. W., Duffy, J. J., and Gerner, E. W. Heat shock proteins in thermotolerance and other cellular processes. Cancer Res., *47:* 5249–5255, 1987.
8. Welch, W. J. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and diseases. Physiol. Rev., *72:* 1063–1081, 1992.
9. Fisch, P., Malkovsky, M., Kovats, S., Sturm, E., Braakman, E., Klein, B. S., Voss, S. D., Morrissey, L. W., DeMars, R., Welch, W. J., Bolhuis, L. H., and Sondel, P. M. Recognition by human Vγ9/Vδ2 T cells of a groEL homolog on Daudi Burkitt's lymphoma cells. Science (Washington DC), *259:* 1269–1273, 1990.
10. Wei, Y-Q., Zhao, X., Kariya, Y., Fukata, H., Teshigawara, K., and Uchida, A. Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res., *54:* 4952–4957, 1994.
11. Ullrich, S. J., Robinson, E. A., Law, L. W., Willingham, M., and Appella, E. A. A mouse tumor-specific transplantation antigen is a heat shock related protein. Proc. Natl. Acad. Sci. USA, *83:* 3121–3125, 1986.
12. Tamura, Y., Tsuboi, N., Sato, N., and Kikuchi, K. 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. J. Immunol., *151:* 5516–5524, 1993.
13. Kaufmann, J. H. E. Heat shock proteins. Immunol. Today, *11:* 126–129, 1990.
14. Ferrarini, M., Pupa, S. M., Zocchi, M. R., Rugarli, C., and Menard, S. Distribution of HSP72 and monomeric laminin receptor expression in human lung cancers infiltrated by γ/δ T lymphocytes. Int. J. Cancer, *57:* 486–451, 1994.
15. Udono, H., and Srivastava, P. K. Heat shock protein 70-associated peptides elicit specific cancer immunity. J. Exp. Med., *178:* 1391–1396, 1993.
16. Van Buskirk, A., Crump, B. L., Margoliash, K., and Pierce, S. K. A peptide binding protein having a role in antigen presentation is a member of the HSP 70 heat shock family. J. Exp. Med., *170:* 1759–1809, 1989.
17. Rippman, F., Taylor, W. R., Rothbard, J. B., and Green, N. M. A hypothetical model for the peptide binding domain of HSP70 based on the peptide binding domain of HLA. EMBO J., *10:* 1053–1059, 1991.
18. Sargent, C. A ., Dunham, I., Trowsdale, J., and Campbell, R. D. Human major histocompatibility complex contains genes for the major heat shock protein. Proc. Natl. Acad. Sci. USA, *86:* 1968–1972, 1989.
19. Born, W., Hall, L., Dallas, A., Boymel, J., Shinnick, T., Young, D., Brennan, P., and O'Brien, R. Recognition of a peptide antigen by heat shock reactive γ/δ-T lymphocytes. Science (Washington DC), *249:* 67–69, 1990.
20. Rajasekar, R., Sim. G., and Augustin, A. Self heat shock and γ/δ T-cell reactivity. Proc. Natl. Acad. Sci. USA, *87:* 1767–1771, 1990.
21. Fu, Y., Cranfill, R., Vollmer, M., Zee, R., O'Brien, R. L., and Born, W. *In vivo* response of murine γδ T cells to a heat shock protein-derived peptide. Proc. Natl. Acad. Sci. USA, *90:* 322–326, 1993.
22. Hamburger, A. N., and Salmon, S. E. Primary bioassay of human tumor stem cells. Sciences (New York), *29:* 461–462, 1977.
23. Bauer, H. C., and Hurtenbach, U., Murine cortical brain cells are autoantigenic from a distinct developmental stage onwards. J. Neuroimmunol., *12:* 1–13, 1986.
24. Berd, D., Herlyn, M., Koprowski, H., and Mastrangelo, M. J. Flow cytometric determination of the frequency and heterogeneity of expression of human melanoma-associated antigens. Cancer Res., *49:* 6840–6844, 1989.